Journal of Medical Virology 57:91–99 (1999)

A New Cluster of Hepatitis A Infection in Hemophiliacs Traced to a Contaminated Plasma Pool

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Recently, several clusters of hepatitis A have been observed among hemophiliacs linked to factor VIII concentrates treated for virus inactivation solely with the solvent/detergent (S/D) method, a procedure that does not affect nonenveloped viruses such as the hepatitis A virus (HAV). A new outbreak of hepatitis A in six hemophiliacs treated with the same lot of a factor VIII preparation occurred recently in Germany. The objective of the study was to clarify whether these diseases were caused by the administration of the S/D-treated plasma product, rather than a community-acquired infection. Polymerase chain reactions designed to detect HAV nucleic acid have been carried out in the implicated factor VIII lots, in the corresponding plasma pools, and in serum samples of four out of six infected individuals. The nucleic acid sequences were determined in samples that resulted in positive amplification products. HAV sequences were found in one of the two plasma pools used for manufacture of the incriminated product, in the incriminated lot itself, and in all recipient sera tested so far, although the latter were collected up to 7 weeks after the onset of jaundice. The sequences obtained were completely identical, revealing a unique HAV strain of genotype IA. This study provides conclusive evidence that hepatitis A can be transmitted by factor VIII concentrates treated solely by the S/D procedure for virus inactivation. This inactivation method is not effective against nonenveloped viruses. Since a number of hepatitis A transmission episodes have been described with such preparations during the past 10 years, their continued use seems to be questionable unless additional virus removal or inactivation steps are introduced to prevent the transmission of nonenveloped viruses. Molecular approaches again proved to be reliable tools for elucidating the chain of virus transmission. ***J. Med. Virol. 57: 91–99, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: hepatitis A virus; HAV transmission; S/D-treated factor VIII; nucleic acid sequence

INTRODUCTION

Hepatitis A virus (HAV) is transmitted usually by the fecal-oral route. However, it is generally accepted that a viremic phase can be observed in early stages of the disease. This viremia may result in parenteral transmission of HAV, which is a rare event [Seeburg et al., 1981; Barbara et al., 1982; Skidmore et al., 1982; Hollinger et al., 1983; Noble et al., 1984; Sheretz et al., 1984; Azimi et al., 1986; Rosenblum et al., 1991; Lee et al., 1992].

Prior to 1988, there were no reports of HAV transmission by fractionated plasma products, including coagulation factor concentrates. It was supposed that any virus would be neutralized by high levels of HAV antibodies present in the pooled plasma. However, clusters of HAV infections of hemophilia patients treated with factor VIII concentrates containing little or no immunoglobulin were reported in Italy [Mannucci et al., 1994], Germany [Gerritzen et al., 1992], Ireland [Temperley et al., 1992; Johnson et al., 1995], Belgium [Peerlinck and Vermylen, 1993], South Africa [Kedda et al., 1995], United States [Anonymous, 1996], and Great Britain (data not shown) between 1988 and 1996.

The manufacturing process of these factor VIII preparations included a solvent/detergent (S/D) treatment for virus inactivation. This method affects reliably lipid-enveloped viruses, such as the human immunodeficiency virus, hepatitis B virus, or hepatitis C virus. By contrast, nonenveloped viruses, such as HAV or human parvovirus B19, are resistant to this procedure.

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Accepted 11 June 1998

TABLE I. Anti-HAV–Negative Patients Treated With Factor VIII Lot #1[a]

Patient	Age at the time of initial infusion	Administered factor VIII units	First infusion[b]	Last infusion[c]	Symptoms of disease	First detection of IgM anti HAV	Date of serum collection
P1	44	12,000	12/11/1996	0/1/29/1997	01/18/1997	01/20/1997	02/20/1997
P2	35	60,000	11/21/1996	01/27/1997	01/08/1997	01/13/1997	02/19/1997
P3	60	44,000	11/26/1996	01/29/1997	01/03/1997	01/13/1997	02/20/1997
P4[d]	46	120,000	11/27/1996	01/29/1997	01/17/1997	01/17/1997	02/28/1997
P5	25	60,000	12/05/1996	01/30/1997	01/13/1997	01/20/1997	04/07/1997
P6[e]	21	4,000	12/02/1996		mid-12/1996	03/20/1997	03/20/1997
P7[e,f]	43	17,000	12/06/1996	01/28/1997	none	ND[g]	04/01/1997
P8[f]	43	2,000	01/06/1997	01/29/1997	none	ND	ND
P9[f]	24	18,000	12/20/1996	01/29/1997	none	ND	ND

[a]The anti-HAV antibody status before factor VIII treatment was negative for all patients, and no other risk factors for an HAV infection were known.
[b]Date of handing over lot #1; first infusion may have taken place some days later.
[c]Date of return of remaining dosages; last infusion may have taken place some days earlier.
[d]In-patient treatment (surgery of the knee) from 12/02 to 12/20/1996; at that time the patient was treated with another factor VIII concentrate.
[e]Patient with von Willebrand's disease.
[f]HAV vaccination on the 01/31/1997 (P7) and 2/11/1997 (P8 and P9).
[g]ND = not done.

HAV RNA has been detected by reverse transcription–polymerase chain reaction (RT-PCR) in factor VIII concentrates prepared in this way [Normann et al., 1992; Mannucci et al., 1994; Kedda et al., 1995; Anonymous, 1996] and was suspected as the source of transmission in these outbreaks. In many cases, however, it was difficult to demonstrate direct links between blood products and HAV infection. Sequence identity of HAV complementary DNA (cDNA) in factor VIII lots and hemophilic recipients treated with these lots was demonstrated only in some cases [Mannucci et al., 1994; Kedda et al., 1995; Anonymous, 1996]. However, in other studies, molecular data were inconclusive and animal infectivity experiments have been negative [Bodemer et al., 1995; Johnson et al., 1995].

We describe an outbreak of hepatitis A among patients of two hemophilia centers in Germany, which occurred in early 1997. Five patients with hemophilia A and one with von Willebrand's disease were involved. All received the same lot of S/D-treated factor VIII preparation. The investigations included the implicated plasma pools and the corresponding lots of factor VIII preparations as well as the sera available at the time of the study from four out of six infected recipients. The data reveal the source of contamination and corroborates the hypothesis that S/D-treated factor VIII concentrates can transmit hepatitis A.

MATERIALS AND METHODS

Studies Samples

Patients studied. In January 1997, five patients with hemophilia A and one with von Willebrand's disease developed typical symptoms of hepatitis, including jaundice, and were found to be IgM anti-HAV–positive. Between November 1996 and January 1997, these patients were treated for their bleeding disorder exclusively with a lot (#1) of factor VIII concentrate that had only been treated with S/D. Anti-HAV antibodies before factor VIII treatment were not found in any of the patients. Sera from four patients (P1 to P4) were available for molecular analysis at the time of this study. These samples were sent, on request, to the Paul-Ehrlich-Institut, the German Competent Authority for vaccines, by the manufacturer of the implicated factor VIII batch. There were three additional susceptible patients (anti-HAV–negative, P7–P9) treated with the same factor VIII lot, who did not develop HAV infection. The patients' data as reported to the Paul-Ehrlich-Institut are summarized in Table I.

Factor VIII preparation. The plasma used for the manufacture of the factor VIII concentrate implicated in the hepatitis A cases was obtained from European sources as well as from plasmapheresis centers in the United States. About 3,000 plasma donations were pooled and cryoprecipitated. Usually, cryoprecipitates of two plasma pools were used as starting material for a single factor VIII lot. The product was purified by ion exchange chromatography and treated by an S/D procedure (tri-[n-butyl] phosphate/tween) for virus inactivation [Horowitz et al., 1985]. Two plasma pools (A and B) were used for the manufacture of the implicated factor VIII lot #1. Moreover, one of the two plasma pools (A) was used for the preparation of another lot of factor VIII (#2), which had not yet been released on the market, but was included in the study.

HAV RNA Detection and Sequencing

Amplification and sequencing of VP3 C terminus and VP1/2A junction. Plasma pools and serum samples were stored at −70°C. The lyophilized coagulation concentrates were reconstituted with half the volume of the provided suspension fluid. The attenuated HAV strain HM-175 (adapted to grow on MRC-5 cells with a titer of 1.6×10^5 $TCID_{50}$/ml indirectly determined by antigen enzyme immunoassay) was used as a positive control. RNA was extracted from 140 μl with the "QIAamp Viral RNA Kit" (QIAGEN, Hilden, Germany) and eluted from the spin columns with 50-μl

RNase-free water. cDNA synthesis and PCR were carried out with outer antisense primer (2226: 5′-CTCCAGAATCATCTCC-3′, numbering as in Cohen et al. [1987b]) and outer sense primer (2020: 5′-ACAGGTATACAAAGTCAG-3′) from the C terminus of the VP3 region.

The sequences of the nested inner primer were as follows: 5′-CACATCAGAAAGGTGAGT-3′ (2039, sense) and 5′-TGTGTAGTAACATCCATA-3′ (2206, antisense). The final incubation volume was 50 μl, containing the following components: 10 μl of the sample RNA, 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM (each) of dNTP, 15 pmol (each) sense and antisense primer, 10 units RNasin (PE Applied Biosystems, Weiterstadt, Germany), 10 units MuLV reverse transcriptase (PE Applied Biosystems), and 1.25 units AmpliTaq Gold DNA polymerase (PE Applied Biosystems). Reaction mixtures were overlaid with 50 μl mineral oil to prevent evaporation.

After a preincubation step at 25°C for 10 min, the samples were incubated at 43°C for 30 min, followed by one cycle of denaturation (and inactivation of reverse transcriptase as well as activation of AmpliTaq Gold) at 95°C for 10 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. Afterwards, 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and a final elongation step of 7 min at 72°C were carried out. The second PCR was undertaken with 2 μl of the first reaction product in a 50-μl reaction mixture containing 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM (each) of dNTP, 1 μM of each internal primer, 0.1 mg/ml bovine serum albumin (New England BioLabs, Beverly, MA), 0.001% gelatin, and 1.25-units AmpliTaq Gold DNA polymerase. The reaction was run with one cycle at 95°C for 10 min, 50°C for 1 min, and 72°C for 1 min, followed by 39 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final elongation step of 7 min at 72°C. The amplified DNA was separated on an agarose gel and visualized by ethidium bromide staining on a UV illuminator.

Procedures designed to avoid contamination were strictly applied throughout the studies [Kwok and Higuchi, 1989]. The HAV region spanning the VP1/2A junction is more variable than the carboxy terminal region of VP3, providing a finer tool for distinguishing one strain from another. RT-PCR was performed by adding outer antisense primer (3398: 5′-AGTAAAA-ACTCCAGCATCCATTTC-3′) and outer sense primer (2891: 5′-GGTTTCTATTCAGATTGCAAATTA-3′). The sequences of the nested primer pair were 2940: 5′-TTTAGTTGTTATTTGTCTGTC-3′ (inner sense) and 3284: 5′-CATTATTTCATGCTCCTCAG-3′ (inner antisense). The conditions for both reactions were the same as those described above for the VP3 region. Sequencing with the 2940 primer resulted in about 300 nucleotides of the VP1/2A junction. For DNA sequencing, an aliquot of the amplified PCR product was directly cloned into the pCR2.1 vector with TA cloning system under the conditions suggested by the manufacturer (Invitrogen BV, Leek, the Netherlands). Plasmid DNA with an insert of the expected size was sequenced with the inner sense primer by using the 373 DNA Sequencer Stretch Line (PE Applied Biosystems).

Amplification and sequencing of 5′ NTR and VP1 amino terminal region. Primers used for amplification of this region were 5′-TCTGTCTTCTTTCTTC-CAGGGCTC-3′ (215 sense) and 5′-ATGAGAGTCAGTC-CTCCGGCGT-3′ (520 antisense) for external amplification, and 5′-GGGACACAGATGTTTGGAACGTCAC-3′ (319 sense) and 5′-ACAACTCACCAATATCCGCC-GCTGT-3′ (480 antisense) for nested amplification. After extraction of viral RNA as described above, reverse transcription (GIBCO-BRL, Bethesda, MD) to cDNA was carried out at 42°C with random hexamers (GIBCO-BRL) in a reaction containing 75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.3) and 200 μM dNTP. The cDNA (20 μl) was added to 30 μl of PCR cocktail containing 300 nM of the external primers, 200 μM dNTP, 1 unit Taq polymerase (Perkin-Elmer, Norwalk, CT), 60 mM KCl, 2.6 mM $MgCl_2$, and 24 mM Tris-HCl (pH 8.3). After the initial denaturation at 94°C for 1.5 min, cycling conditions included annealing at 50°C for 30 sec, extension at 72°C for 30 sec, and denaturation at 94°C for 30 sec for 30 cycles. Nested amplification using 1/50 of the first round product was undertaken in a buffer containing 50 mM KCl, 200 μM dNTP, 3 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.3) as described above with the exception that the annealing temperature was 52°C.

The conditions for RNA extraction, reverse transcription, and heminested PCR amplification of the VP1 amino terminal region were carried out as previously described [Soucie et al., 1998]. Products were purified by acrylamide gel separation. ABI Prism Dye Terminator Cycle sequencing was performed using both the internal sense and antisense primers. After removal of excess primer, the products were analyzed using an ABI 373 sequencer (Applied Biosystems, Foster City, CA).

Analysis of Genetic Relatedness Between HAV Strains

Nucleotide sequences were aligned by using PILEUP. The phylogenetic tree was constructed from the distance matrix by the neighbor-joining method using the program CLUSTREE [Saitou and Nei, 1987].

RESULTS

Epidemiological Information

In spite of recommendations that hemophiliacs without anti-HAV antibodies should be vaccinated or given factor VIII concentrate that included steps that inactivate nonenveloped viruses, in the period from November 1996 to January 1997, nine patients without anti-HAV were treated for their bleeding disorder exclusively with the lot #1 of a factor VIII concentrate that had only been treated with S/D for virus inactivation (Table I). Six of these patients (P1 to P6) developed typical symptoms of hepatitis A. None reported contact with persons with jaundice or known to have hepatitis

TABLE II. Detection of HAV RNA by RT-PCR in Plasma Pools, in Factor VIII Preparations, and in Serum of Hemophiliacs

Sample	Number of RNA extractions	HAV RT-PCR Region VP3 N_{pos}/N_{tot}[a]	HAV RT-PCR Region VP1/2A N_{pos}/N_{tot}[a]
Plasma pool A	5	7/11	4/6
Plasma pool B	4	0/8	0/6
Factor VIII, lot #1	6	6/17	4/4
Factor VIII, lot #2	3	2/6	1/3
Patient P1	4	0/6	4/6
Patient P2	4	4/6	1/6
Patient P3	4	3/6	5/6
Patient P4	1	1/2	2/2

[a]Number of positive runs/total number of runs.

TABLE III. Nucleic Acid Sequence Variability of HAV cDNA

Sample	Identity to consensus sequence Number of nucleotides per clone (number of sequenced clones) VP3 region (122 nucleotides)	VP1/2A region (286 nucleotides)
Plasma pool A	122 (8)	286 (2); 285 (2)[a]
Factor VIII, lot #1	122 (7)	286 (2); 285 (1)[b]
Factor VIII, lot #2	122 (2)	286 (2); 285 (1)[c]
Patient P1	Not done	286 (5); 285 (1)[d]
Patient P2	122 (4)	286 (3)
Patient P3	122 (6)	286 (4)
Patient P4	Not done	286 (3)
Positive PCR control	122 (4)	286 (4)

[a]Nucleotide could not be clearly identified (both at nucleotide position 3048, no clear fluorescent peak).
[b]Nucleotide position 3011: C for T.
[c]Nucleotide position 3266: C for A.
[d]Nucleotide position 3190: C for T.

A, travel to countries in which HAV is highly endemic, or consumption of foods suspected to be contaminated with HAV. No increased incidence of HAV was observed in the areas where the patients live. Sera from four patients (P1 to P4) available at that time were included in the study.

HAV RNA Detection

In preliminary experiments, the sensitivity of the HAV PCR procedure was determined by using the titrated HAV strain HM-175. The virus stock was diluted in HAV negative plasma. The detection limit was 0.0045 $TCID_{50}$/PCR assay. Samples of factor VIII concentrates were spiked with a definite amount of the cell culture-adapted strain to check for effects inhibitory to the RT-PCR. However, no inhibition could be observed. The inclusion of the attenuated HM-175 strain as a low positive control (0.045 $TCID_{50}$/ml) in every experiment provided verification of successful amplification.

HAV RNA was detected clearly by RT-PCR with two different sets of nested primers, spanning the carboxy terminal region of VP3 or the VP1/2A junction of the HAV genome, in plasma pool A, in both lots (#1 and #2) of the factor VIII preparation, and in all tested sera of hemophilia patients, who were treated with lot #1 of the factor VIII preparation and who developed hepatitis A (Table II). No amplicons could be generated by RT-PCR from the second plasma pool that was also used for the production of factor VIII lot #1. The amplification results reflect the very low HAV titer in the samples. On account of the Poisson distribution of small numbers of target molecules, some runs did not result, as expected, in a detectable amplification (Table II). Therefore, several PCR runs had to be carried out in order to test an aliquot of each sample large enough to detect a low virus load reliably [Chudy et al., 1995]. The negative controls included in each experiment gave negative amplification results in all cases.

The detection of HAV sequences in the sera of the hemophilia patients who developed hepatitis A was not expected, since the sera were taken more than 40 days after the onset of jaundice. Although anti-HAV IgG antibodies have normally been developed at this stage of the disease, an HAV viremia at a low level could be observed. Unfortunately, samples from earlier bleeding dates (e.g., when blood was drawn for anti-HAV IgM tests) were not available for this study.

HAV Sequencing

Nested PCR is a highly sensitive method for detecting minute amounts of nucleic acids, although there is always a risk of cross-contamination. We sequenced and compared amplicons obtained from the patients' sera, the implicated plasma pool, the factor VIII lots, and the positive run controls. A number of clones were sequenced per sample. The sequence of the VP3 region (122 nucleotides) from different clones of the same sample was identical (Table III). A nucleotide exchange in one position of the VP1/2A region (286 nucleotides) was observed in three clones (out of 12 total) for three different samples (Table III). One nucleotide at the same position could not be identified in two out of four clones from the plasma pool A for technical reasons (lack of resolution). However, the sequencing results revealed that the error rate of the AmpliTaq polymerase due to the lack of a proofreading activity was not deleterious for the precise amplification of these fragments. On the other hand, no great sequence variability within an HAV strain would be expected.

The sequences from sera of all four hemophilia patients tested were identical with both amplified HAV regions. There was 100% identity between these sequences and those amplified from the lots #1 and #2 of the factor VIII concentrate and the implicated plasma pool, respectively (Figs. 1 and 2). In comparison with these sequences, the cDNA sequence of the attenuated HM-175 strain used as positive control showed 7 nucleotide exchanges for the VP3 region and 30 exchanges for the amplified region of the VP1/2A junction.

The genotype and uniqueness of this strain compared to other known strains of HAV was confirmed by

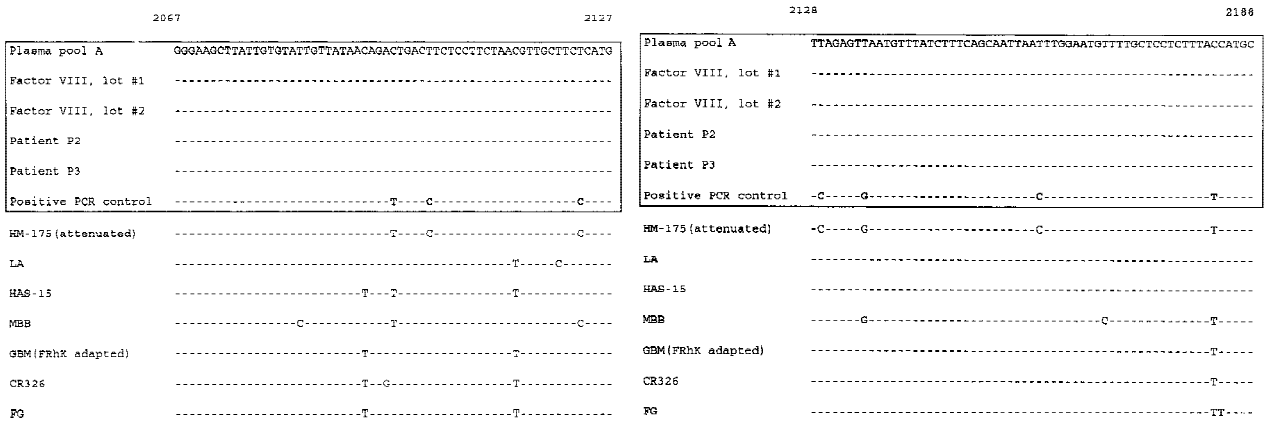

Fig. 1. Comparison of HAV nucleotide sequences at the C terminus of the VP3 region (nucleotides 2067–2188) derived from amplified genomes of different samples (boxed sequences) with known HAV strains. GenBank accession number of the strains used for comparison: attenuated HM-175 (M16632), LA (K02990), HAS-15 (X15464), MBB (M20273), GBM (X75214), CR326 (M10033), and FG (X83302). Dashes represent homology. Differences are shown by appropriate base letter. Nucleotides are numbered according to the wild-type HM-175 HAV sequence [Cohen et al., 1987b].

sequencing the amino terminal region of VP1 (nucleotides 2208–2355) of the amplification product from one patient (P3); in addition, the sequence of the 5′ end (nucleotides 331–460) from the precursor plasma pool A, lot #1, and one of the patients (P3) was also identical and most closely related to sequenced genotype IA strains, supporting transmission of the virus (data not shown).

Analysis of Genotype of Amplified HAV Sequences and Their Genetic Relation to Other Sequences

The relatively conserved regions within the 5′ NTR and near the carboxy terminus of VP3 provide the opportunity of successful primer binding and efficient amplification of unknown HAV sequences. By contrast, the region spanning the VP1/2A junction is more variable, providing a finer tool for distinguishing different strains [Robertson et al., 1992, 1994]. The HAV sequence deduced from the implicated factor VIII lots, the corresponding plasma pool, and patients' sera is unique within the VP1/2A region (nucleotides 2979–3264) when compared to other strains. It shows the highest nucleic acid sequence homology (98.6%) to the strains LA and HAS-15. All belong to genotype IA (BLAST Search, National Center for Biotechnology Information, Bethesda, MD; data not shown). The phylogenetic tree analysis reveals a closer link to the strain LA (Fig. 3). It should be noted that previous analyses of the genetic relations between HAV strains included only a 168-nucleotide segment at the VP1/2A junction (nucleotides 3024–3191) [Robertson et al., 1992, 1994]. Inclusion of a longer segment in the analysis increases the reliability of the observed degree of the genetic relationship.

The results rule out decidedly the possibility of laboratory contamination, as the sequence of the unique strain differs from that of the positive PCR control by 10.5% of the nucleotide positions. The attenuated HM-175 strain belongs to genotype IB. The remarkable distance between the two sequences is reflected in the dendrogram (Fig. 3). The sequence of the positive control differs from the published sequence at two base positions (nucleotide 3027: A instead of T; and nucleotide 3196: A instead G) within that region [Cohen et al., 1987a]. These base exchanges lead to amino acid substitutions: Thr for Ser (VP1-274) and Asn for Ser (2A-30), respectively. In addition, positive amplification products and sequencing in three different laboratories further support the uniqueness of this strain and argue against laboratory contamination.

The complete identity of the sequences from the plasma pool A, the two lots of the factor VIII preparation, and the sera of the hemophilia patients who developed hepatitis A confirm the hypothesis that the hepatitis A infections in six hemophiliacs originated from contaminated blood products. This contamination can be traced back to plasma pool A.

DISCUSSION

An outbreak of HAV infection is reported among patients with hemophilic disorders who were treated with a factor VIII preparation, the manufacture of which included solely the S/D procedure as a specific virus inactivation step. No increase in HAV incidence was observed in the area where the patients live, and no other risk factors for HAV infection were identified. Instead, HAV sequences were amplified by RT-PCR from samples included in the chain of this hepatitis A transmission event.

The results obtained with the hemophilia patients' sera provide more data regarding the length of the period of hepatitis A viremia. In the cases investigated, HAV was still present, even though the clinical symptoms had already disappeared. Based on the data from the pre-PCR era, it is usually believed that viremia lasts for 2 to 4 weeks during the presymptomatic phase of an HAV infection [Havens, 1946; Krugman et al.,

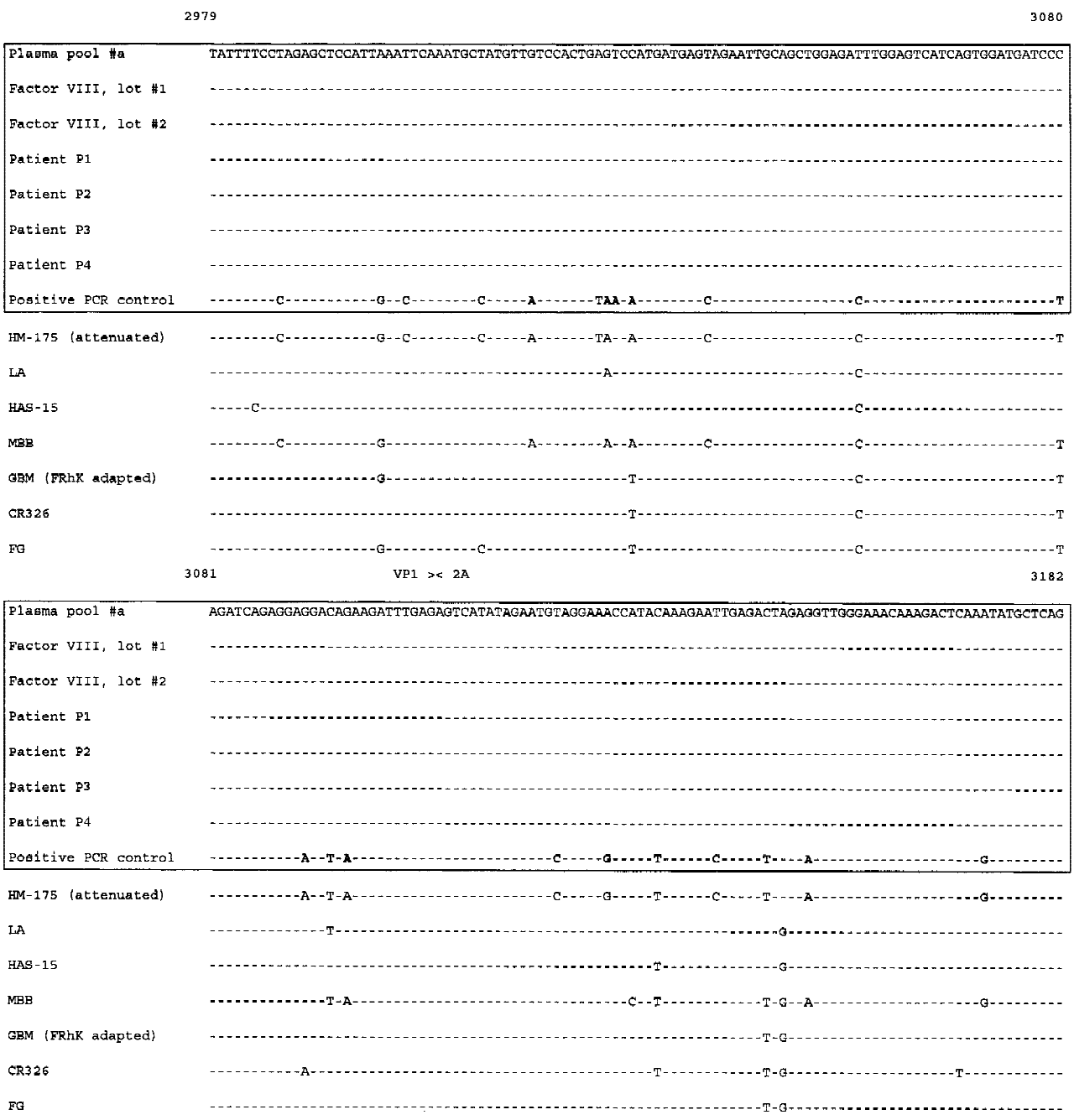

Fig. 2. Comparison of HAV nucleotide sequences at the VP1/2A junction (nucleotides 2979–3264) derived from amplified genomes of different samples (boxed sequences) with known HAV strains. See legend to Figure 1.

1962; Giles et al., 1964; Ward and Krugman, 1982; Hollinger et al., 1983]. The finding that HAV could still be detected 7 weeks after the onset of jaundice were corroborated both by a report on an HAV epidemic, which occurred between June and September 1995 in Marburg, Germany [Kaul, 1996], and by studies undertaken with sera of Italian hemophiliacs [Mannucci et al., 1994].

Nested PCR is a sensitive and powerful technique that allows the detection of low-level virus contaminations. In these circumstances, a positive control is necessary to demonstrate the absence of inhibitory substances, but the potential for contamination during handling is present. The sequencing of positive amplicons provides proof of the specificity of the reaction. Also, the successful amplification of four separate genomic regions of HAV in three different laboratories argues against a contamination.

We have clearly demonstrated the identity of the sequences derived from the amplified HAV cDNA of (1) one of the implicated plasma pools, (2) the factor VIII lots produced from this pool, and (3) serum of hemo-

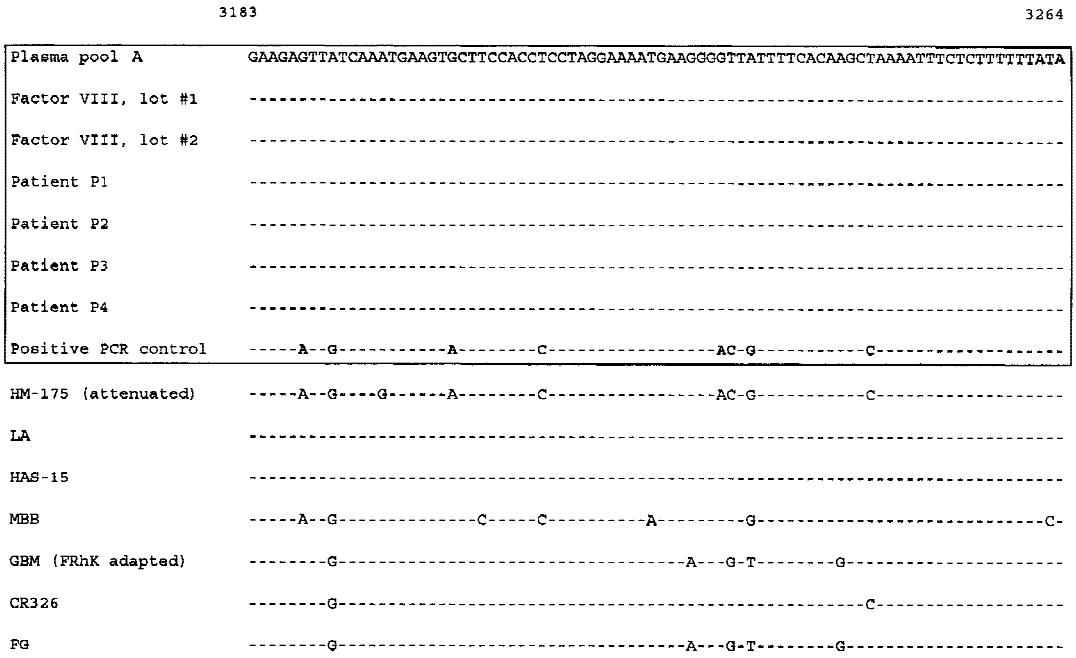

Fig. 2. Continued

philia patients who developed hepatitis A after treatment with this clotting factor concentrate. Plasma pool A proved to be the source of the HAV contamination. It consisted of plasmapheresis donations collected in the United States. The results of the molecular analysis provide evidence that HAV was transmitted to recipients by lot #1 of the implicated factor VIII preparation.

The sequencing results make it very unlikely that the contaminated plasma pool or the factor VIII lots contained multiple HAV strains. A single routine screening of the contaminated plasma pool for HAV by RT-PCR may not have been successful, as HAV RNA was present only in limiting dilution. Assuming a Poisson distribution, the virus titer can be calculated from the PCR results obtained with the VP3 region (Table II, assuming the RNA extraction to be 90% efficient and the cDNA synthesis to be 20% efficient). On that basis, the virus titer was calculated to be approximately 6×10^2 genomes/ml in the plasma pool A and approximately 3×10^2 copies/ml in the factor VIII lot #1. Such a titer could be explained by a single viremic donation with a titer of approximately 2×10^6/ml, which entered a plasma pool of approximately 3,000 donations. We know that an HAV load calculated by PCR results reflects all physical HAV particles (assuming that 1 genome equivalent corresponds to 1 physical virus particle), but only a part of these may be infectious.

In the plasma pool A, an anti-HAV titer of 6.11 International Units/ml was determined by the manufacturer. In the final product, no antibodies against HAV could be detected by commercial enzyme immunoassays. The reasons why HAV was not neutralized completely and removed as *virus*/antibody complexes are unknown. It is unlikely that there was a unique failure in good manufacturing practices during the production of factor VIII lot #1 since the second lot (#2) prepared from the same plasma pool was also HAV RNA–positive, and the deduced nucleic acid sequences were identical to those of the other samples. The factor VIII lot #2 has not been released on the market.

Data on the maximum and minimum HAV quantities that may be removed during manufacture of high-purity factor VIII concentrate by anion exchange chromatography and by S/D treatment have been calculated [Lemon, 1994, 1995; Lemon et al., 1994]. Although the sum of the reduction factors estimated for the different production steps (IgG-mediated neutralization, cryoprecipitation, S/D procedure, chromatography, and lyophilization) seem to support a high margin of safety, our data demonstrate that HAV was able to pass from the plasma pool to the final product. From this observation, it was possible to conclude that the reduction factors for single steps may not be additive in this case. It is also obvious that the single steps do not make a crucial contribution to the HAV safety of this factor VIII preparation.

So far, there have been no reports of successful in vivo or in vitro infectivity experiments with PCR-positive factor VIII preparations [Bodemer et al., 1995; Johnson et al., 1995]. This may be explained by the very low virus load in the factor VIII lot, which could result in an uneven distribution of viruses in individual vials of the final product, and by the wide discrepancy between a virus titer calculated by PCR and a titer measured by infectivity assays. For the cell culture adapted strain of HM-175, the ratio between infectious and physical HAV particles was determined to be approximately 1 to 200. Therefore, the inoculum used in unsuccessful infectivity experiments may not have contained sufficient amounts of infectious HAV particles.

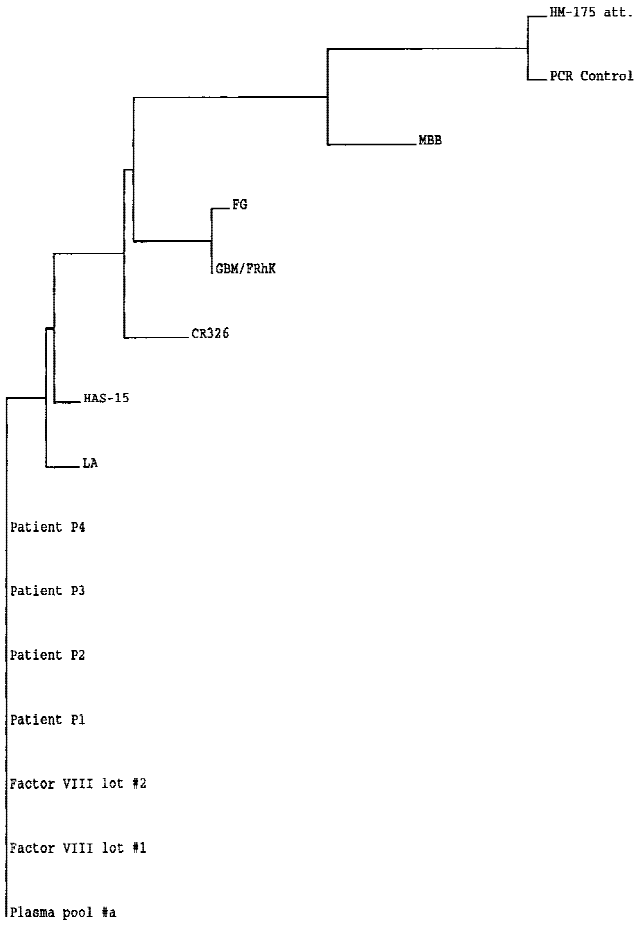

Fig. 3. Genetic relationship between HAV strains of genotype I. The dendrogram derived from CLUSTREE illustrates the comparison of nucleotide sequences within a 286-base segment at the VP1/2A junction. See legend to Figure 1.

It is difficult to calculate the infectious dose for those patients who developed hepatitis A after treatment with factor VIII lot #1, because details (volume of infusions, time course) were not accessible to us. Patient 6 developed HAV infection after a bolus infusion of only 4,000 units of factor VIII. This corresponds to a virus amount of approximately 10^4 HAV particles (3×10^2 genomes/ml = 3×10^2 genomes per 100 units). It should be kept in mind that the coagulation factor concentrates are applied intravenously, which is not the usual route of HAV infection.

It seems difficult to design an animal transmission study since large amounts of factor VIII preparation have to be administered in order to demonstrate infectivity. Nevertheless, the manufacturer in question has already initiated animal studies with the implicated factor VIII lot. There are indications that at least one of the treated animals developed hepatitis A (data not shown).

The results of this study obtained by molecular approaches and supported by epidemiological data provide clear evidence that HAV can be transmitted by highly purified factor VIII preparations, which include an S/D treatment as the only specific virus inactivation step. This transmission was due to the inclusion of a viremic plasma unit in the starting material. These findings indicate that appropriate steps for the inactivation of nonenveloped viruses, in addition to S/D inactivation, should be added to the manufacturing process in order to ensure the safety of highly purified factor VIII preparations. Consideration should be given to discontinue the use of such factor VIII preparations, which rely solely on the S/D treatment for virus inactivation.*

ACKNOWLEDGMENTS

We are indebted to Martin Selbert for his excellent technical assistance of sequencing, and to Ina Plumbaum for her help with the manuscript. Moreover, the authors would like to acknowledge Dr. Thomas Gärtner of the Georg-Speyer-Haus, Frankfurt, Germany, for developing the conditions for amplification of the 5′ NTR products and providing them for sequencing.

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*Since acceptance of this article, the findings obtained with the sera of patients 6 and 7 have confirmed the results of this study.

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